

# Single Amino Acid Differences Are Sufficient for CD4<sup>+</sup> T-Cell Recognition of a Heterologous Virus by Cattle Persistently Infected with Bovine Viral Diarrhea Virus

Trevor Collen,<sup>\*,1</sup> Alastair J. Douglas,<sup>†</sup> David J. Paton,<sup>‡</sup> Gang Zhang,<sup>§</sup> and W. Ivan Morrison<sup>\*</sup>

<sup>\*</sup>Division of Immunology and Pathology and <sup>§</sup>Division of Molecular Biology, Institute for Animal Health, Compton, Berkshire RG20 7NN, United Kingdom; <sup>†</sup>Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Stormont, Belfast BT4 3SD, Northern Ireland; and <sup>‡</sup>Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey KT15 3NB, United Kingdom

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Cattle that are persistently infected (PI) with one strain of bovine viral diarrhea virus (BVDV) can resolve infection with a second, antigenically heterologous strain but not the homologous strain. Since CD4<sup>+</sup> T cells are thought to be critical for the resolution of acute BVDV infection (Howard *et al.*, 1992, *Vet. Immunol. Immunopathol.* 32, 303–314), we have examined the recognition of a heterologous virus (NADL) by CD4<sup>+</sup> T cells from Pe515-PI animals. The immune response of non-PI control cattle challenged with NADL or Pe515ncp was strain cross-reactive, whereas Pe515-PI animals responded to NADL only. The immune repertoire of both groups included NS3, which differs by approximately 1% (9/683) amino acids between these two viruses. Lymphoproliferative responses to proteins and synthetic peptides corresponding to three nonconservative differences in NS3 demonstrated that CD4<sup>+</sup> T cells from non-PI control animals responded well to proteins but poorly to the peptides from both viruses. In contrast, PI animals were responsive to heterologous proteins and peptides but nonresponsive to the homologous equivalents. A single amino acid difference between the two sequences was sufficient to allow responsiveness. © 2000 Academic Press

**Key Words:** bovine viral diarrhea virus; persistent infection; heterologous challenge; immunological nonresponsiveness; CD4<sup>+</sup> T-cell repertoire; B-cell repertoire; NS3 proteinase; synthetic peptides.

## INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a positive-sense RNA virus of the genus *Pestivirus* belonging to the family *Flaviviridae* (Wengler, 1991). Two biotypes of the virus, noncytopathic (ncp) and cytopathic (cp), are distinguished by their effects in tissue culture. BVDV infection of naïve cattle generally results in either asymptomatic or mild clinical symptoms and is characterized by transient viremia, nasal shedding, and leukopenia followed by the production of neutralizing antibodies (Malmquist, 1968). In recent years, cases of a severe and sometimes fatal acute disease associated with genotype II ncp-BVDV infections have been reported (Bolin and Ridpath, 1992; Pellerin *et al.*, 1994).

Persistent infection (PI) can arise when a fetus is infected by ncp-BVDV *in utero* during the first trimester of pregnancy (i.e., prior to immunological incompetence). Such animals remain lifelong carriers and usually do not produce antibody to the persisting virus. Experimental superinfection of PI animals with antigenically similar or identical (homologous) cp virus results in an acute, in-

variably fatal condition known as mucosal disease (MD) and the animals die without making antibody against the challenge virus (Bolin *et al.*, 1985a; Brownlie *et al.*, 1984). These observations gave rise to the suggestion that PI animals are immunologically tolerant to the persisting virus (Bolin *et al.*, 1985b; Brownlie *et al.*, 1984; Coria and McClurkin, 1978; Larsson and Fossum, 1992; McClurkin *et al.*, 1984). That this putative tolerance is specific to the persisting virus is suggested by the finding that PI animals can resolve infections with antigenically dissimilar (heterologous) isolates (Bolin *et al.*, 1985b; McClurkin *et al.*, 1984). However, formal evidence of tolerance to the persisting virus and a definition of the mechanism are lacking.

The invariable isolation of pairs of ncp and cp viruses from animals that die from MD (Greiser-Wilke *et al.*, 1993; McClurkin *et al.*, 1985; Moennig *et al.*, 1990; Wilhelmssen *et al.*, 1991) suggested that antigenically homologous cp virus arises by recombination or by mutation of the ncp genome. Examples of recombination events that have been characterized are reviewed in Meyers and Thiel (1996). However, MD can also occur following heterologous or semihomologous challenge (Bolin *et al.*, 1985a,b; Bruschke *et al.*, 1998; Fray *et al.*, 1998; Fritzemeier *et al.*, 1997; Ridpath and Bolin, 1995; Shimizu *et al.*, 1989). In this situation, the disease has a delayed onset and the

<sup>1</sup> To whom correspondence and reprint requests should be addressed at Institute for Animal Health, Department of Immunology and Pathology, Compton, Berkshire RG20 7NN, United Kingdom. Fax: +44-1635-577263. E-mail: [trevor.collen@bbsrc.ac.uk](mailto:trevor.collen@bbsrc.ac.uk).

TABLE 1  
Virus Isolation from Nasopharyngeal Swabs and Blood Samples

Animal	Persistent virus	Challenge virus	Virus isolation	
			Blood	Nasal swab
51/96	n.a. <sup>a</sup>	Pe515ncp	+ <sup>b</sup> (5, 7, 12)	+ (2, 5, 7, 9, 12, 14, 17)
5334	n.a.		+ (5, 7, 9, 14)	+ (5, 7, 9, 12, 14, 17)
582	n.a.	NADL	+ (12)	+ (5, 12)
591	n.a.		+ (14)	+ (12)
5172	Pe515ncp	NADL	+ (5, 9)	+ (5, 7, 9)
32/96	Pe515ncp	NADL	+ (9, 12)	+ (5, 12)
33/96	Pe515ncp	NADL	+ (9)	+ (5, 12)
36/96	Pe515ncp	NADL	— <sup>c</sup>	—

<sup>a</sup> n.a. = not applicable (i.e., non-PI).

<sup>b</sup> + = virus detected. Numbers in parentheses represent the day(s) when sampling was positive for challenge strain biotype (NADL = cytopathic, Pe515ncp = noncytopathic).

<sup>c</sup> — = challenge virus not detected.

animals produce neutralizing antibodies against the challenge virus prior to the onset of MD. Low levels of neutralizing antibody against the homologous virus have also been reported (Brock *et al.*, 1998; Collins *et al.*, 1999; Edwards *et al.*, 1991), although it is unclear whether this response is against the homologous virus or an adventitious heterologous infection. Both early- and delayed-onset MD are consistent with the same model for pathogenesis but differ in that the delayed form takes a finite but variable time for the generation of homologous cp virus to occur.

Infection of non-PI cattle results in the production of neutralizing antibodies that confer protection against reinfection. Acquisition of maternal antibody by PI calves can reduce virus titers but does not clear the PI virus (Howard *et al.*, 1989). The preceding observations suggest that antibody alone is insufficient to effect the resolution of BVDV infection. Studies involving *in vivo* depletion of T-cell subsets have demonstrated that disease resolution in BVDV naïve, non-PI calves is strongly dependent on the CD4<sup>+</sup> T-cell population (Howard *et al.*, 1992). The repertoire and effector functions of CD4<sup>+</sup> T cells have still to be determined with respect to clearance of BVDV.

In this study we have examined the ability of CD4<sup>+</sup> T cells from Pe515-PI calves to recognize proteins and peptides from the homologous ncp/cp virus pair and a defined heterologous cp challenge utilizing experimentally produced, gnotobiotically derived PI animals housed in high disease security accommodation. We demonstrate that the PI calves are nonresponsive to the homologous virus *in vitro*, both at the level of neutralizing antibody and lymphoproliferation, and extend these observations to demonstrate CD4<sup>+</sup> T-cell response to NS3 of the challenge virus. Responses to synthetic peptides indicate that limited sequence differences between the

homologous and the heterologous viruses can be sufficient for T-cell recognition of the heterologous virus strain.

## RESULTS

### Clinical status of animals

Prior to experimental infection, all of the animals were tested on two occasions at 3-week intervals for the presence of BVDV-specific antibody and viremia. None of the animals had detectable circulating neutralizing antibody and only the four PI animals were viremic ( $10^{7.0 \pm 0.7}$  TCID<sub>50</sub>/ml). The four PI animals and two non-PI animals were challenged with NADL and the other two non-PI animals were infected with Pe515ncp. Following challenge, ncp virus was isolated from all of the PI calves and the two Pe515ncp-infected, non-PI animals, and cp virus was isolated from three of the PI calves (5172, 32/96, and 33/96) and the two NADL-infected, non-PI animals (Table 1). At the peak of viremia the viral burden was  $10^{6.9 \pm 0.7}$  TCID<sub>50</sub>/ml ncp virus for the Pe515ncp control animals,  $10^{6.3 \pm 0.6}$  TCID<sub>50</sub>/ml cp virus for the NADL control animals, and  $10^{5.3 \pm 0.5}$  TCID<sub>50</sub>/ml cp virus for the PI calves. Virus was recovered more frequently from nasal secretions than from blood and all animals cleared the challenge virus by 19 days postinfection (d.p.i.).

### Heterologous challenge of PI cattle induces specific immunity with restricted antibody specificity

All of the animals seroconverted by 14 d.p.i. following experimental challenge. Cross-neutralizing antibodies reactive with Pe515ncp and NADL were detected in the serum of the non-PI animals (5334, 51/96, 582, 591). Pe515-PI animals produced neutralizing antibodies that reacted with NADL but not Pe515ncp (5172, 32/96, 33/96) (Fig. 1).

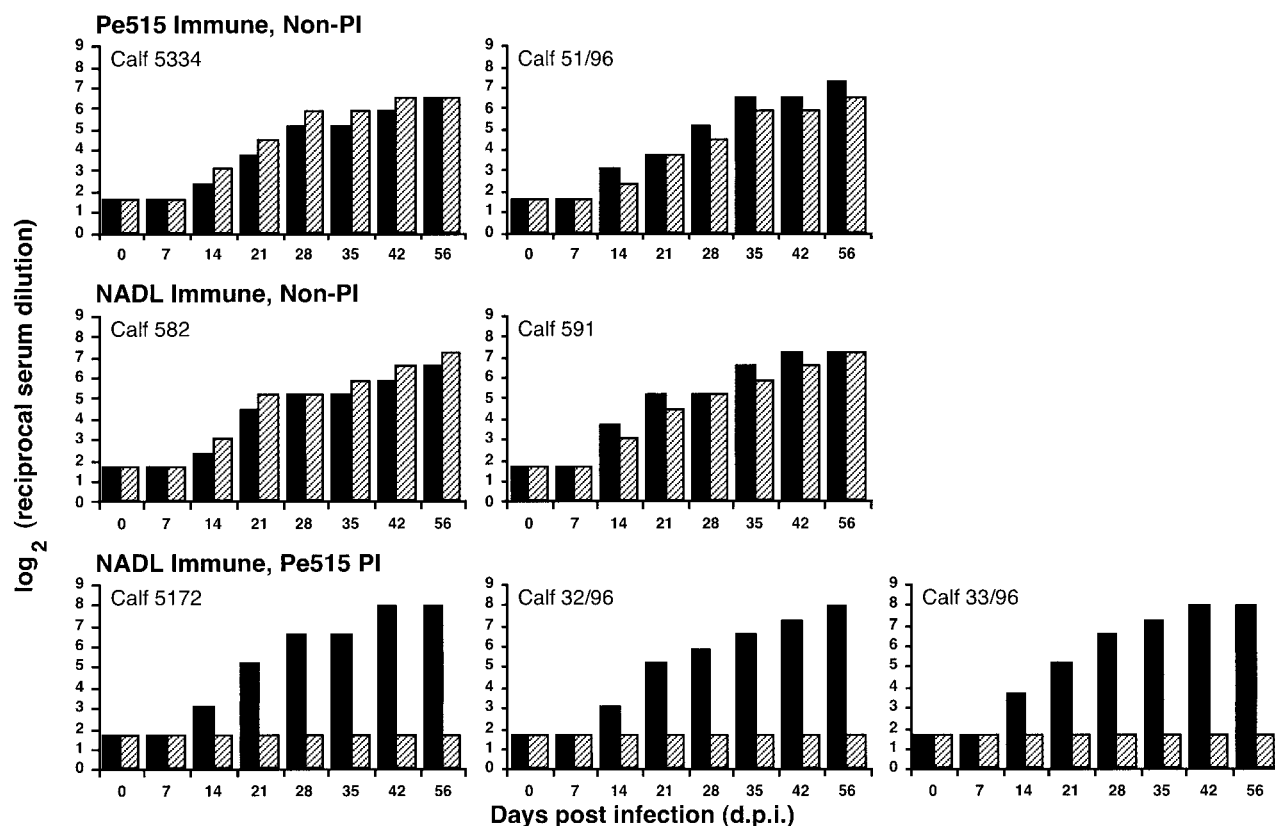


FIG. 1. Virus-neutralizing activity of sera collected from non-PI cattle challenged with either NADL (582, 591) or Pe515ncp (5334, 51/96) and Pe515ncp-PI cattle challenged with NADL (5172, 32/96, 33/96). Sera were collected at various times after infection and assayed against 100 TCID<sub>50</sub> of either Pe515ncp (hatched bars) or NADL (filled bars).

The specificity of antibody was examined by immunoprecipitation/Western blot analysis of NADL and Pe515cp antigens. Precipitation of Pe515 proteins was carried out against Pe515ncp and Pe515cp (homologous pair). Only the results for Pe515cp are presented to show NS3 as a discrete viral product. Representative results from a non-PI animal (591) and a PI animal (5172) are shown in Fig. 2. At 56 d.p.i., antibodies from the non-PI animals recognized a range of viral proteins from Pe515cp and NADL. Interestingly, the protein profiles obtained using Pe515cp and NADL antigens grown for 24 h were very similar (Fig. 2, lanes 1 and 2), as were the profiles obtained using Pe515cp grown for 24 or 40 h (data not shown). However, the NS2-3 band obtained when using 40-h NADL lysate was fainter than that for the 24-h lysate (data not shown). Serum antibodies from the PI animals recognized none of the Pe515cp proteins and only one of the NADL proteins. Of the PI animals, only animal 5172 was kept for more than 84 d.p.i. Serum antibodies sampled at 84 d.p.i. (data not shown) and 112 d.p.i. (Fig. 2) were found to recognize both NADL and Pe515 viruses with respect to E2, NS3, and NS2-3 (Fig. 2, lanes 5 and 6). Homologous neutralizing antibody activity was not detected at any time point.

These data confirm that PI animals are capable of

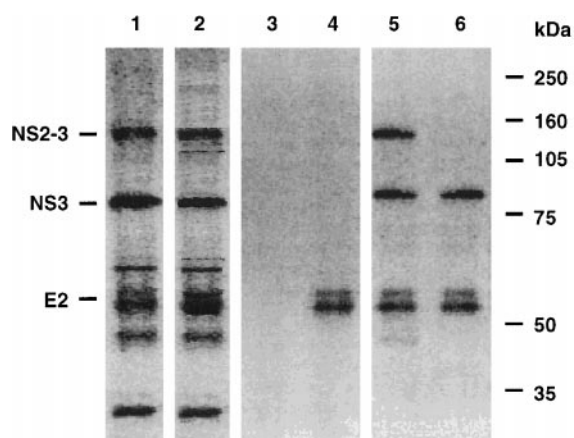


FIG. 2. Immunoprecipitation/Western blot analysis of virus proteins reacted with serum obtained from non-PI and Pe515ncp-PI calves challenged with NADL. Viral proteins were immunoprecipitated using 56 and 112 d.p.i. serum and resolved by 12.5% SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose, probed with biotinylated anti-BVDV IgG, and detected by ECL. The figure shows representative data from a non-PI calf (591) at 56 d.p.i. (lanes 1 and 2) and a PI calf (5172) at 56 d.p.i. (lanes 3 and 4) and 112 d.p.i. (lanes 5 and 6) for Pe515cp antigen (lanes 1, 3, and 5) or NADL antigen (lanes 2, 4, and 6). Precipitation of Pe515 proteins was carried out against Pe515ncp and Pe515cp, but only the results for Pe515cp are presented to show NS3 as a discrete viral product.

making an antibody response following challenge with a heterologous BVDV isolate and that, at least up to Day 56 after challenge, the antibody produced is largely specific for the challenge virus.

#### **CD4<sup>+</sup> T cells from heterologously challenged PI cattle proliferate in response to the challenge virus but not persisting-virus antigens**

To determine whether Pe515 proteins were antigenic in Pe515-PI cattle, PBMC from non-PI and PI animals were assayed at weekly intervals against varying concentrations of mock and BVDV antigens in proliferation assays.

The maximum difference between mock and viral antigens was obtained using a 1:40 dilution of antigen, and under these conditions non-PI cattle responded to Pe515ncp by 42–56 d.p.i. and to NADL by 21 d.p.i. Pe515-PI cattle challenged with NADL also responded to NADL antigen by 21 d.p.i. The four non-PI cattle responded to both Pe515 and NADL antigen, whereas the three PI cattle responded to NADL only (Figs. 3 and 4).

PBMC from PI animals secrete live virus into assay cultures. To test whether the presence of live ncp virus influenced the ability of PBMC to respond to sonicated antigens, PBMC from non-PI animals were cocultured with 1:40 mock, Pe515ncp, or NADL antigen in the presence of 0, 0.05, 0.5, 5, or 20 m.o.i. of infectious Pe515ncp. The results indicated that there was an increased proliferative response to sonicate antigens that was maximal at 0.5 m.o.i. (24%). The addition of live virus did not adversely affect proliferative responses to sonicate antigens at any of the m.o.i.'s tested (results not shown).

The sonicated viral antigens used to screen T-cell responses comprise a complex mixture of viral and cellular proteins of which viral proteins are a relatively minor component. To test whether proliferative responses were influenced by the purity of viral antigen, PBMC from non-PI and PI cattle were stimulated with varying concentrations of beads carrying affinity-captured proteins. Figure 3 shows representative responses of non-PI and PI animals. Responses were stimulated by antigens captured with monoclonal antibodies (MAbs) specific for E<sup>rns</sup>, E2, and NS3. The non-PI animals responded to proteins from both viruses, whereas the PI animals responded to NADL only. The antigen dilutions that gave a positive response extended over a greater range than for the crude sonicate antigens ( $6.25 \times 10^3$ – $1 \times 10^5$  beads/well) (data not shown).

The MAbs used for the immunocapture of BVDV antigens are specific for discrete viral proteins. However, Western blot analysis of affinity-captured antigen indicated that the immunocaptured preparations contained a predominance of the target protein but that they were not pure. Thus, E<sup>rns</sup> and E2 were present in the antigens

prepared with either the E2- or E<sup>rns</sup>-specific MAbs, respectively. Similarly, antigen prepared with the NS3-specific MAb contained both NS3 and the precursor protein NS2-3. To further examine the response to these proteins, NADL and Pe515cp proteins were resolved by SDS-PAGE and prepared as nitrocellulose-bound antigens. Only NS3 was resolved to a discrete band. Figure 4 shows that PBMC from the non-PI cattle were stimulated by nitrocellulose-bound NS3 from both viruses, whereas PBMC from the PI cattle were stimulated by NS3 from NADL only. All three PI calves responded to the NS3 (80-kDa) preparation and two of the animals (5172 and 33/96) responded to a fraction corresponding to  $48\text{--}53 \pm 4$  kDa (data not shown). Control responses against equivalent fractions from mock-infected antigen were negative, although slightly higher than medium alone.

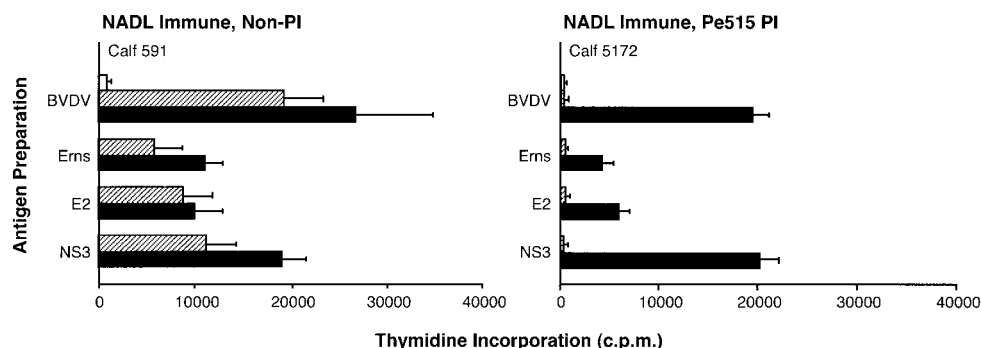
These data demonstrate that PI animals are capable of making a T-cell response following challenge with a heterologous BVDV isolate and confirm nonresponsiveness to homologous virus proteins.

#### **CD4<sup>+</sup> T cells from heterologously challenged PI cattle can recognize single amino acid differences between challenge and persistent virus NS3 proteinase**

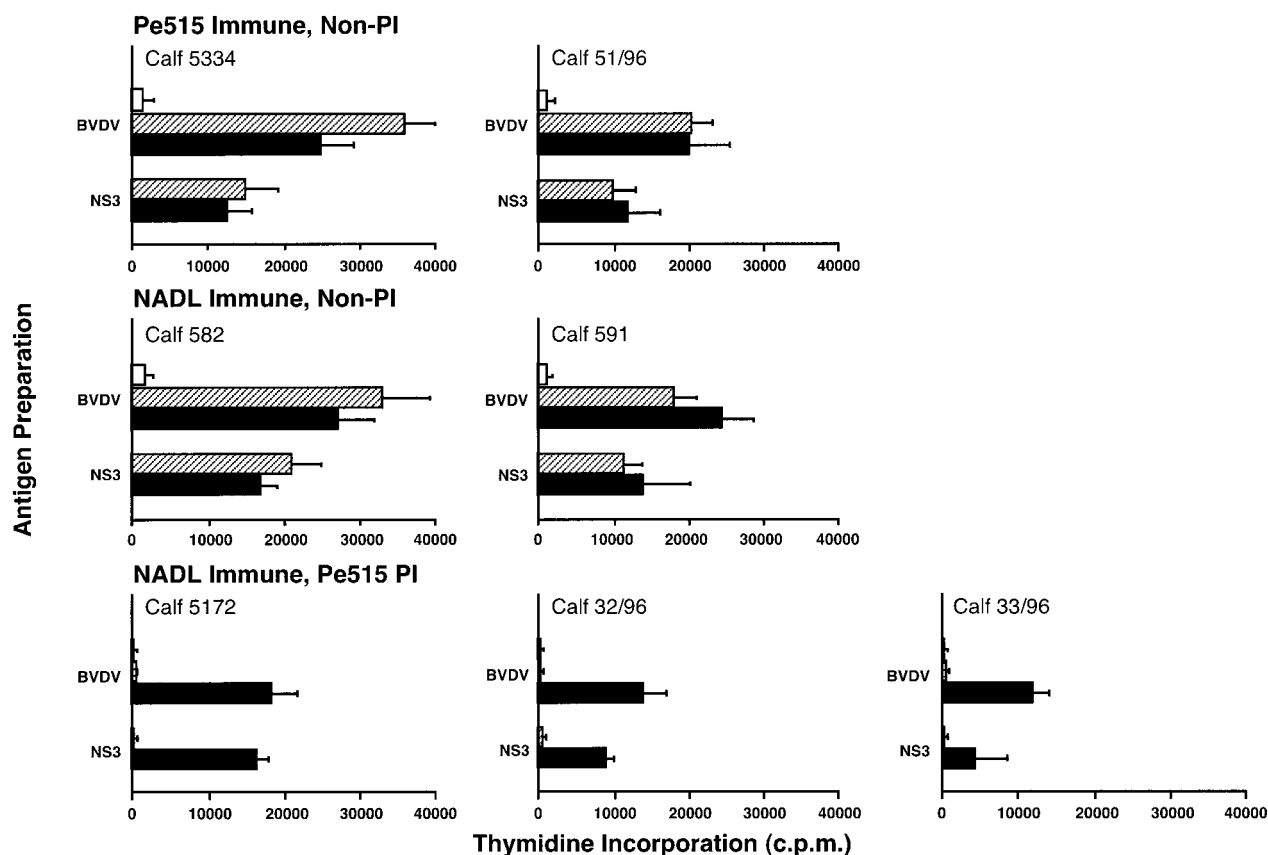
NADL-immune, Pe515-PI cattle were responsive to NS3 from NADL but nonresponsive to NS3 from Pe515. To determine that 1% sequence variation between the proteins was sufficient to account for responsiveness to the heterologous NS3, PBMC were stimulated with varying concentrations of chemically synthesized peptides in proliferation assays.

A pair of peptides that correspond to a single substitution at position 1892 (R  $\leftrightarrow$  T) (p1 and p2, Table 2) and a second pair of peptides that correspond to two closely located substitutions at positions 2353 (G  $\leftrightarrow$  S) and 2360 (T  $\leftrightarrow$  A) (p3 and p4, Table 2) were tested for recognition by T cells from the Pe515-PI and non-PI control cattle. PBMC from the three NADL-immune, Pe515-PI cattle responded to the NADL-derived peptide p3 (NADL: 2343–2369) but not to the equivalent Pe515ncp-derived peptide p4 (Pe515: 2343–2369) (Fig. 5). In contrast, weak responses, as defined by proliferation at 10  $\mu\text{g/ml}$  but not at 2  $\mu\text{g/ml}$  or lower, were observed against the Pe515-derived peptide p4 for the Pe515ncp-immune, non-PI animal 5334, and against the NADL-derived peptide p3 for the NADL-immune, non-PI animal 591 (Fig. 5).

A C-terminally truncated version of p3, peptide p5 (NADL: 2343–2359), which contained only the position 2353 (G  $\leftrightarrow$  S) substitution, was recognized by Pe515-PI calf 5172 with similar magnitude and dose kinetics to that of the longer peptide, p3. An N-terminally truncated version of p3, peptide p7 (NADL: 2343–2369), which overlapped with p5 by six residues and contained only the



**FIG. 3.** Proliferation by PBMC in response to enriched BVDV proteins. BVDV proteins from NADL or Pe515cp were captured on paramagnetic beads coated with monoclonal antibodies specific for E<sup>ns</sup>, E2, or NS3, as described under Materials and Methods. The figure shows representative data from a non-PI calf (591) and a PI calf (5172) assayed at 35 d.p.i. PBMC ( $1 \times 10^5$ /well) were stimulated with mock antigen (1:40 final dilution), BVDV antigen (1:40 final dilution), or enriched antigen ( $5 \times 10^4$  beads/well) prepared from mock (open bars), NADL (filled bars), or Pe515cp (hatched bars) antigens and cultured for 6 days at 37°C. Responses are presented as the geometric means ( $\pm 1$  SE) of c.p.m. (591: medium only, 1209; PWM, 121,981; 5172: medium only, 78; PWM, 118,673).



**FIG. 4.** Proliferation by PBMC in response to nitrocellulose-bound NS3 antigen. Nitrocellulose-bound NS3 was prepared from NADL or Pe515cp and used to stimulate PBMC from non-PI calves challenged with either Pe515ncp (5334, 51/96) or NADL (582, 591), and three Pe515ncp-PI calves challenged with NADL (5172, 32/96, 33/96) at 42 and 63 d.p.i. for the NADL- or Pe515-infected cattle, respectively. PBMC ( $1 \times 10^5$ /well) were incubated with a 1:40 final dilution of mock antigen, BVDV antigen, or nitrocellulose-bound NS3 prepared from mock (open bars), NADL (filled bars), or Pe515cp (hatched bars) antigens and cultured for 6 days at 37°C. Responses are presented as the geometric means ( $\pm 1$  SE) of c.p.m. (582: medium only, 1198; PWM, 97861; 591: medium only, 1301; PWM, 122,298; 51/96: medium only, 1282; PWM, 102,761; 5334: medium only, 1173; PWM, 128,851; 5172: medium only, 87; PWM, 117,815; 32/96: medium only, 112; PWM, 101,811; 33/96: medium only, 71; PWM, 87,413).

TABLE 2

Synthetic Peptides Representing Amino Acid Sequences from NS3<sup>a</sup> of NADL and Pe515ncp Strains of BVDV

Virus	Peptide No.	Peptide Sequence <sup>a</sup>	Difference
NADL	p1	....1880.....1890. SGIQTVSKNRADLTEMVKK	R <sub>1882</sub>
Pe515ncp	p2	SGIQTVSKNTADLTEMVKK	T <sub>1882</sub>
NADL	p3	....2350.....2360..... DPGNQVVETGKALKQVTGLSSAENAL	G <sub>2353</sub> /T <sub>2360</sub>
Pe515ncp	p4	DPGNQVVETSKALKQVAGLSSAENAL	S <sub>2353</sub> /A <sub>2360</sub>
NADL	p5	DPGNQVVETGKALKQV	G <sub>2353</sub>
Pe515ncp	p6	DPGNQVVETSKALKQV	S <sub>2353</sub>
NADL	p7	KALKQVTGLSSAENAL	T <sub>2360</sub>
Pe515ncp	p8	KALKQVAGLSSAENAL	A <sub>2360</sub>

<sup>a</sup> Peptides synthesised according to the sequences published for NADL (Collett *et al.*, 1988) and Pe515 (Meyers *et al.*, 1992). Sequence numbering is given with respect to the numbering used for NADL in Collett *et al.* (1988).

position 2360 (T ↔ A) substitution, was only weakly recognized. Weak responses were also observed for the other two Pe515-PI animals with peptides p1 (NADL:

1873–1891), p3, p5, and p7 (Figs. 5 and 6) and for non-PI calf 591 with peptides p5 and p7.

Taken together, these data confirm the induction of

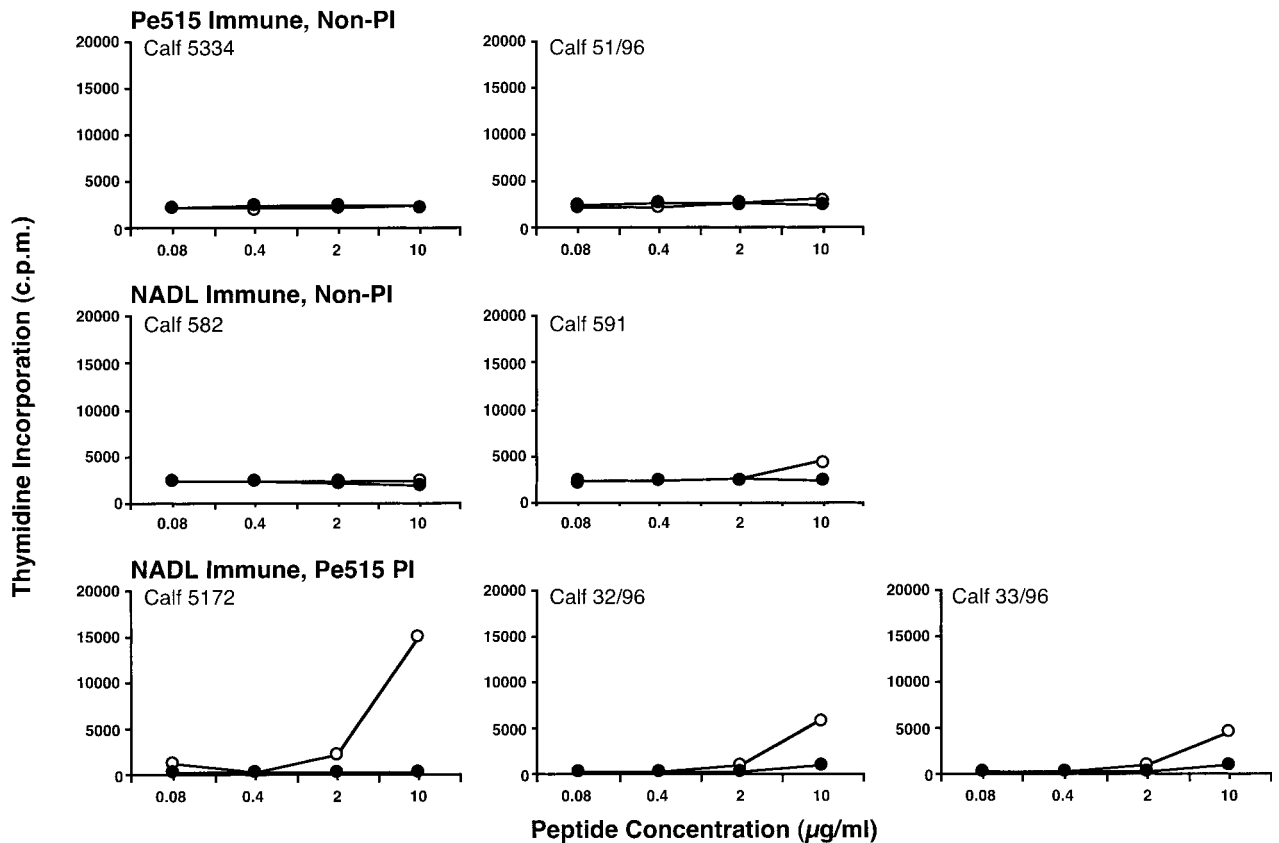


FIG. 5. Proliferation by PBMC in response to synthetic peptides. Synthetic peptides corresponding to the sequence of NS3 (Table 2) at selected amino acid differences between Pe515ncp (open symbols) and NADL (closed symbols) were used to stimulate PBMC from non-PI calves challenged with either Pe515ncp (5334, 51/96) or NADL (582, 591), and three Pe515ncp-PI calves challenged with NADL (5172, 32/96, 33/96) at 49 and 70 d.p.i. for the NADL- or Pe515-infected cattle, respectively. PBMC ( $1 \times 10^5$ /well) were incubated with peptide (○, p1; ●, p2) over a concentration range from 10 to 0.08  $\mu$ g/ml and cultured for 6 days at 37°C. Responses are presented as the geometric means of c.p.m. (582: medium only, 1209; PWM, 87,442; 591: medium only, 1279; PWM, 92,312; 51/96: medium only, 1165; PWM, 84,212; 5334: medium only, 1063; PWM, 81,201; 5172: medium only, 84; PWM, 76,532; 32/96: medium only, 91; PWM, 71,520; 33/96: medium only, 89; PWM, 84,223).



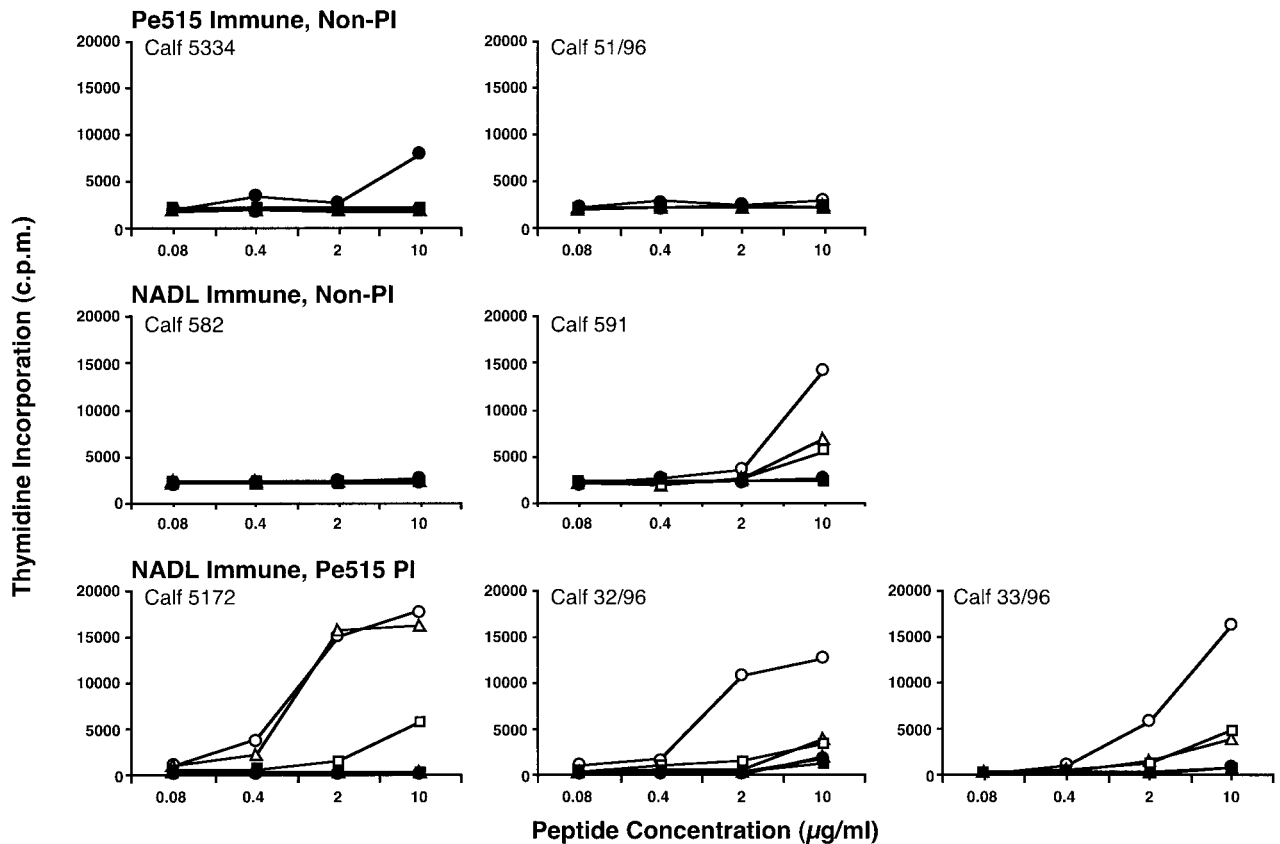


FIG. 6. Proliferation by PBMC in response to synthetic peptides. Synthetic peptides corresponding to the sequence of NS3 (Table 2) at selected amino acid differences between Pe515ncp (open symbols) and NADL (closed symbols) were used to stimulate PBMC from non-PI calves challenged with either Pe515ncp (5334, 51/96) or NADL (582, 591), and three Pe515-PI calves challenged with NADL (5172, 32/96, 33/96) at 49 and 70 d.p.i. for the NADL- or Pe515-infected cattle, respectively. PBMC ( $1 \times 10^5$ /well) were incubated with peptide ( $\circ$ , p3;  $\bullet$ , p4;  $\triangle$ , p5;  $\blacktriangle$ , p6;  $\square$ , p7;  $\blacksquare$ , p8) over a concentration range from 10 to 0.08  $\mu\text{g/ml}$  and cultured for 6 days at  $37^\circ\text{C}$ . Responses are presented as the geometric means of c.p.m. (582: medium only, 1209; PWM, 87,442; 591: medium only, 1279; PWM, 92,312; 51/96: medium only, 1165; PWM, 84,212; 5334: medium only, 1063; PWM, 81,201; 5172: medium only, 84; PWM, 76,532; 32/96: medium only, 91; PWM, 71,520; 33/96: medium only, 89; PWM, 84,223).

NS3-reactive T cells following acute heterologous BVDV infection of PI animals and demonstrate that the immune repertoire of PI animals can exploit minimal or even single amino acid differences to distinguish between the homologous and heterologous viruses.

## DISCUSSION

The primary aim of this study was to establish an experimental model that would allow us to examine nonresponsiveness to BVDV in PI cattle. BVDV-PI calves were generated experimentally using a cloned ncp isolate of Pe515 (Brownlie *et al.*, 1984) and the resulting animals were housed in disease-secure accommodation. *In vitro* comparison of B-cell and T-cell responses of PI and non-PI animals to a heterologous virus challenge demonstrated that the  $\text{CD4}^+$  T-cell repertoire of PI animals is tolerant to the homologous virus and gave an indication of the extent of sequence difference required for  $\text{CD4}^+$  T cells to discriminate between the homologous and heterologous virus antigens.

Persistently infected animals and strain-specific non-responsiveness to ncp-BVDV arise through *in utero* infection prior to the fetus's becoming immunocompetent. When PI animals are challenged postnatally by homologous cp virus, it is speculated that tolerance to the PI virus is responsible both for the failure of these animals to produce neutralizing antibody and for uncontrolled replication and viral cytopathology (Bolin *et al.*, 1985b; Brownlie *et al.*, 1984; McClurkin *et al.*, 1984). This model is supported by the invariable recovery of antigenically homologous ncp/cp virus pairs from animals that have died from mucosal disease (MD) (Greiser-Wilke *et al.*, 1993; McClurkin *et al.*, 1985; Moennig *et al.*, 1990; Wilhelmssen *et al.*, 1991). However, antigenic analyses of viruses isolated from field cases of MD and experimental infections using uncloned viruses indicate that MD can also be induced by heterologous viruses (Bolin *et al.*, 1985b; Bruschke *et al.*, 1998; Fray *et al.*, 1998; Fritzemeier *et al.*, 1997; Ridpath and Bolin, 1995; Shimizu *et al.*, 1989). In this situation, MD has a delayed onset and the ani-

mals produce neutralizing antibodies against the challenge virus prior to the onset of MD. Both early- and delayed-onset MD are consistent with the same model for pathogenesis but differ in that the delayed form takes a finite but variable time for the generation of homologous cp virus. In the short-term, challenge with a heterologous virus generally leads to resolution of infection, production of antibodies to the challenge virus (Bolin *et al.*, 1985a; McClurkin *et al.*, 1984), and, occasionally, low levels of antibodies to homologous virus (Brock *et al.*, 1998; Collins *et al.*, 1999; Edwards *et al.*, 1991).

It has been suggested that the lack of antibody production to the homologous virus by PI cattle is the result of B-cell tolerance (Larsson *et al.*, 1990; Malmquist, 1968). In our study, heterologous infection of PI cattle resulted in seroconversion (Fig. 1) and clearance (Table 1) of the challenge virus without affecting the persisting virus. After challenge, the antibody repertoire of the PI animals was very restricted compared to that of the non-PI cattle (Fig. 2), such that by 56 d.p.i., the non-PI cattle recognized most proteins from both viruses, whereas the PI animals recognized only heterologous E2. Interestingly, at both 84 and 112 d.p.i., serum from PI calf 5172 had reactivity with E2 and NS3 from both viruses. These sera were able to neutralize NADL and precipitate both homologous and heterologous E2, but were nonneutralizing for both biotypes of the Pe515 virus pair. The data described are consistent with other studies in which antibody to homologous PI virus has been detected following heterologous challenge (Bolin, 1988; Brock *et al.*, 1998; Donis and Dubovi, 1987). The production of antibodies reactive with the homologous virus indicates that the B-cell repertoire of PI cattle can include the homologous virus. Activation of B cells specific for homologous virus, albeit under undefined circumstances, suggests that B-cell tolerance may be incomplete.

Using lymphoproliferation as a correlate of CD4<sup>+</sup> T-cell response (Collen and Morrison, 2000), the CD4<sup>+</sup> T-cell repertoire of non-PI animals was strain cross-reactive, whereas that of the NADL-immune, Pe515-PI animals was NADL specific (Figs. 3 and 4). Responses to enriched proteins from NADL or Pe515cp demonstrated that the CD4<sup>+</sup> T-cell repertoire of non-PI and PI calves included E<sup>ns</sup>, E2, and NS2-3/NS3 and recognition of NS3 was confirmed using protein extracted from SDS-PAGE gels. Importantly, we found no evidence that homologous antigens were recognized at any level of purity tested, suggesting that the nonresponsiveness of the Pe515-PI calves to Pe515-sonicate antigen was not simply a property of the complex protein mixture or the high concentration of cellular proteins. In addition, coculture of sonicate and live virus antigens provided no indication that infectious virus released by PI-PBMC might compromise responses, either directly through infection or by high

zone tolerance. Others have used live virus stimulation to study T-cell responses (Lambot *et al.*, 1997; Larsson and Fossum, 1992). Thus, our data are consistent with the view that the T-cell repertoire of PI animals is tolerant to the homologous PI virus. Nonresponsiveness to homologous antigens was observed for at least 16 weeks post heterologous infection, suggesting that the CD4<sup>+</sup> T-cell tolerance is complete.

NADL was used to challenge the PI animals because (1) it is antigenically heterologous to Pe515, (2) it is the basis of an attenuated live vaccine licensed for use in North America, and (3) its genome has been fully sequenced (Collett *et al.*, 1988). However, it has been suggested that immune responses to cp and ncp viruses are regulated differently (Lambot *et al.*, 1997, 1998). Thus, the strength of the lymphoproliferative response to NADL, and to NS3 in particular, could reflect a Th1 bias introduced by the choice of challenge virus. However, cattle infected with either Pe515cp or Pe515ncp both recognize heterologous NS3 (Collen and Morrison, 2000; Lambot *et al.*, 1997), demonstrating that lymphoproliferative recognition of NS3 is not dependent on the choice of biotype used for challenge. Thus, heterologous cp challenge is a useful tool for the analysis of the CD4<sup>+</sup> T-cell repertoire of PI cattle, with the caveat that the bias of target antigens and effector responses overall may differ for acute ncp and cp infections or between non-PI and PI cattle and will require further studies.

NS3 of NADL (Collett *et al.*, 1988) and Pe515ncp (Meyers *et al.*, 1992) differ by nine out of 683 amino acids. Responses to synthetic peptides corresponding to three nonconservative substitutions suggested that the PI animals were more responsive than were the non-PI animals to the same viral sequences. Lack of or weak T-cell recognition of determinants might result if (1) test peptides did not correspond to the sequence of the priming virus, (2) the sequences selected are unable to bind the MHC molecules of a particular animal, or (3) the sequences selected represent subdominant or cryptic determinants. In this study, animals were challenged with NADL-Weybridge (Paton *et al.*, 1992) and the animals used were of undefined MHC haplotype. Although there was no prior evidence to suggest that any of the peptides tested would be antigenic, several animals made good responses to one or more of the peptides, suggesting that the poorer responsiveness of the non-PI cattle was not the result of a mismatch between the challenge viruses and peptide sequences.

Furthermore, in a separate study, we have shown that the dominant determinants for two defined MHC class II haplotypes are located in regions that are separate from the substitutions examined in the present study (in preparation). We suggest that the regions examined in the current study constitute subdominant determinants. Interestingly, the dose kinetics of animal 5172 and the



overlapping reactivity of peptides p3 and p5 suggest that residues 2343–2359 contain a T-cell determinant for this animal. In this context, the proline at position –2 could indicate a processing motif such that the G  $\leftrightarrow$  S substitution would fall within the core epitope as defined by Falk *et al.* (1994). For the other two PI animals, peptide p3, but neither p5 nor p7, was recognized. Since peptides p5 and p7 overlap with each other and with p3, we can speculate that the determinant recognized by these animals resides toward the middle of p3 (e.g., neither fully in p5 nor fully in p7). Thus, for these animals either the G  $\leftrightarrow$  S or T  $\leftrightarrow$  A substitutions, or both together, could be responsible for heterologous responsiveness.

We propose the following model to explain tolerance in BVDV-PI animals and how such animals might produce antibody to the persisting virus. During the induction of tolerance, PI-T cells specific for BVDV-infected cells become suppressed as if the BVDV proteins were self-antigens. Antigen-presenting cells (APC) expressing both dominant and subdominant viral T-cell epitopes can act as a bridge to bring locally suppressive, regulatory T cells into close proximity with naïve CD4<sup>+</sup> T cells and thereby cause suppression of the naïve T cells (linked suppression) (Davies *et al.*, 1996; Hoyne *et al.*, 1997; Wise *et al.*, 1998). If the priming antigen is always present, as it is in a persistent virus infection, dominant and subdominant epitopes would both remain under negative regulation and effector responses would be compromised through lack of T-cell help. In contrast, tolerance for the effector cell populations may be incomplete and, given sufficient or appropriate T-cell help, they could be made to respond. Self-tolerance to virus proteins mediated by tissue-specific expression of genes in transgenic mice can be overcome by presentation of that protein in association with foreign T-cell epitopes, provided either through full virus infection or by immunization with a carrier-protein conjugate (Steinhoff *et al.*, 1994). Thus, on challenge with heterologous BVDV, PI cattle can respond by recognizing differences between the persisting and challenge viruses.

Moreover, several studies have demonstrated that CD4<sup>+</sup> T cells specific for one protein of a complex antigen, or an epitope of that protein, can provide help to B cells specific for a different protein of the same complex antigen (Milich *et al.*, 1987; Russell and Liew, 1979; Scherle and Gerhard, 1988). Such help is most efficient when B-cell and T-cell epitopes are closely associated on the same protein but can also occur with decreasing efficiency, as epitopes become separated on the same protein or between proteins present in the same APC. Thus, in the case of BVDV, help for B cells specific for E2 could be provided by CD4<sup>+</sup> T cells specific for NS3, or vice versa, if both are presented by the same APC. Such limitations could account for the highly selective antibody response that we observed in this study (Fig. 2). In

our study, the recognition of synthetic peptides with limited sequence differences suggests that regions of viral proteins with minimal substitutions could be sufficient to provide linked recognition. Clearly, the greater variability of E2 compared to NS3 would greatly increase the probability of such an outcome.

The results reported here highlight the importance of even single amino acid differences between BVDV isolates in overcoming nonresponsiveness in PI cattle. We propose that tolerance of the CD4<sup>+</sup> T-cell repertoire, together with a critical dependence on CD4<sup>+</sup> T-cell help for effector responses, is sufficient to account for the nonresponsive character of PI animals. Although the number of animals in this study is small and the conclusions may be highly speculative, the data support a model for tolerance and responsiveness that can be tested more rigorously.

## MATERIALS AND METHODS

### Viruses

The Pe515 and NADL viruses used in this study are antigenically heterologous, genotype I isolates of BVDV. The Pe515 viruses were a homologous pair of cloned cytopathic (cp) and noncytopathic (ncp) isolates that have been described previously (Brownlie *et al.*, 1984). The NADL isolate was originally obtained from Central Veterinary Laboratories (Weybridge, UK) and then propagated at IAH-Compton (M. C. Clarke, personal communication).

### Antibodies

BVDV-specific monoclonal antibodies WB112 (anti-NS3), WB162 (anti-E2), and WB210 (anti-E<sup>ms</sup>) have been described previously (Edwards *et al.*, 1988; Paton *et al.*, 1991). Biotinylated polyclonal anti-BVDV immunoglobulin (2331-B) was prepared from serum that had a high neutralizing antibody titer, and recognized all major BVDV proteins by immunoprecipitation and immunoblotting. The IgG fraction was purified and concentrated on a Protein G column and biotinylated using sulfo-NHS-LC-biotin (Pierce and Warriner UK, Chester, UK).

### Cells and media

MDBK cells were provided by the Tissue Culture section of this Institute and were cultured in Dulbecco's modified minimal essential medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) that was free from BVDV and BVDV-specific antibody, 2 mM glutamine, 30 mM MgCl<sub>2</sub>, 0.25% (w/v) lactalbumin hydrolysate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 µg/ml amphotericin B (complete DMEM). Maintenance medium was as described for complete DMEM but contained only 2% (v/v) FCS. Bovine PBMC were cultured in RPMI 1640

containing 5% (v/v) heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 25 mM HEPES, 0.05 mM 2-mercaptoethanol, and 10  $\mu$ g/ml gentamicin sulfate (complete RPMI).

## Animals

Two pairs of virus-free, BVDV antibody-negative Friesian calves aged between 4 and 7 months were housed in the medium security accommodation at the Compton Laboratory of the Institute for Animal Health. The calves were infected with  $5 \times 10^6$  TCID<sub>50</sub> of either Pe515ncp (animals 5334 and 51/96) or NADL (animals 582 and 591) by the intranasal route.

The BVDV PI calves used in this study were produced by surgical infection of fetuses. Briefly, six pregnant cows were presented for fetal infection at approximately 60 days of gestation. Following laparotomy, 5 ml of cell culture fluid containing  $10^6$  TCID<sub>50</sub> of Pe515ncp was injected directly into the amniotic fluid. The incision was repaired by standard surgical procedures and routine antibiotic treatment given. PI calves were derived by hysterotomy at approximately 10 days before term and each calf was delivered into a sterile isolator and then transferred to high disease security accommodation. The calves were fed BVDV-free, BVDV antibody-free colostrum within 6 h of birth. Blood samples were collected and the PI status of the calves was confirmed by seeding buffy coat cells onto a permissive cell substrate (MDBK cells) followed 4–5 days later by immunoperoxidase staining to detect viral antigens (Smith *et al.*, 1988). One Friesian PI calf (animal 5172) and three Aberdeen Angus PI calves (animals 32/96, 33/96, and 36/96) were challenged with the NADL strain of BVDV at approximately 4 months of age using the same dose and route as that described for the non-PI animals. Nasal swabs and blood samples were collected at –28, –7, –2, 0, 2, 5, 7, 9, 12, 14, 17, and 21 d.p.i. Each sample underwent one cycle of freezing and thawing before being seeded onto MDBK cell cultures. The virological status of the animals was determined by examination of the cultures for absence of cytopathic effect followed by immunoperoxidase staining to detect viral proteins. Blood samples were collected weekly into preservative-free heparin for the isolation of peripheral blood mononuclear cells (PBMC) to determine the ability of the animals to mount proliferative cell-mediated responses. All of the calves, except Pe515-PI animal 36/96, were successfully infected by the specified challenge virus; 36/96 is not reported further.

## Preparation of mock and viral antigens

BVDV was propagated in MDBK cells overlaid with maintenance medium. The expression of BVDV proteins

in infected cells was confirmed by immunoperoxidase staining using 2331-B as the BVDV probe.

Uninfected (mock antigen) and virus-infected (BVDV antigen) MDBK cells were treated as described previously (Collen and Morrison, 2000). Briefly, 850-cm<sup>2</sup> roller bottles of MDBK cells were infected with BVDV at a multiplicity of infection (m.o.i.) of 0.1 TCID<sub>50</sub>/cell for 90 min at 37°C. Cells were overlaid with maintenance medium and incubated at 37°C with continuous rolling. Infected cells were detached using a cell-scraper (40 h for NADL and 60 h for the Pe515 isolates), washed twice with PBS, and suspended at approximately  $10^8$  cells/ml in ice-cold PBS containing 0.025% glutaraldehyde (Aldrich, Dorset, UK). After 15-min incubation on ice, cells were washed three times with PBS, suspended at  $10^8$  cells/ml in RPMI 1640 medium (without serum), and thoroughly disrupted by sonication (Model W375 Cell Disruptor, Heat Systems, Ultrasonics, Farmingdale, NY). Cell debris was removed by centrifugation at 600 *g* for 10 min and the supernatant aspirated and stored at –80°C in 0.2-ml aliquots (BVDV antigen). Mock-infected cells were incubated with PBS instead of virus and then processed as described for the Pe515-infected cells (mock antigen). A single roller bottle (approximately  $3 \times 10^8$  cells) provided 2.5 ml of antigen at approximately 4 mg/ml of total protein.

## Synthetic peptides

Synthetic peptides representing selected regions of NS3 from NADL and Pe515ncp BVDV were prepared using a BT7400 Multiple Peptide Synthesis system (Cambridge Research Biologicals, Norwich, Cheshire, UK). The sequences chosen for synthesis (Table 2) were selected based on nonconservative amino acid substitutions between the two viruses. Nine amino acids were included on either side of a given substitution to allow for the potential of MHC binding.

## Bead-bound antigen

Viral proteins were affinity captured from sonicated antigen using paramagnetic beads as described by Hawke *et al.* (1992). Briefly,  $4 \times 10^7$  goat anti-mouse IgG beads (M-450; Dynal UK, Merseyside, UK) were washed six times with PBS to remove preservative. The beads were mixed with 50  $\mu$ g anti-BVDV monoclonal antibody diluted to 0.5 ml with PBS and incubated for 18 h at 4°C with continuous rotation. Antibody-coated beads were washed six times with PBS and diluted to  $10^7$  beads/ml in complete RPMI. Beads ( $4 \times 10^6$ ) were incubated with 100  $\mu$ l (approximately 100  $\mu$ g total protein) of BVDV antigen diluted in 1 ml complete RPMI for 30 min at room temperature with continuous rotation. Bead-bound antigen was washed four times with PBS, irradiated with long-wavelength UV light (10,000 joules, 365 nm; Strata-

linker 1800), washed once with PBS, and suspended at  $10^6$  beads/ml in complete RPMI. Bead-bound antigen was stored at 4°C.

### Nitrocellulose-bound antigen

Nitrocellulose-bound antigen was prepared as described previously (Collen and Doel, 1990). Briefly, mock or BVDV antigen was diluted 1:1 with electrophoresis sample buffer (0.2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 62.5 mM Tris, pH 6.8) and heated to 90°C for 5 min. Twenty micrograms total protein/lane was resolved in a 12.5% discontinuous SDS-PAGE system, under reducing conditions, using a Miniprotean II electrophoresis tank (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Proteins were transferred to nitrocellulose using a Trans-blot semidry blotter (Bio-Rad) and blocked with 5% (w/v) semiskimmed milk in PBS containing 0.1% (v/v) NP40. The locations of BVDV proteins were identified on a strip cut from the edge of the membrane by incubation with a 1:1000 dilution of 2331-B as the virus-specific probe followed, after washing, with a 1:1000 dilution of streptavidin-horseradish peroxidase conjugate. Bound conjugate was visualized by the addition of PBS containing diaminobenzidine (DAB, 1 mg/ml; Sigma D5637) and  $\text{H}_2\text{O}_2$  (0.003% v/v) as the chromogen-substrate and NiCl (0.04% w/v) to enhance the color. In the case of p80, the position was confirmed by colocalization with molecular weight markers. Nitrocellulose-bound antigen was prepared from the region of the filter corresponding to  $80 \pm 4$  and  $48\text{--}53 \pm 3$  kDa, respectively, from both BVDV and mock antigen blots.

### Immunoprecipitation/immunoblot analysis

Confluent (90–95%) monolayers of MDBK cells were infected with NADL, Pe515ncp, or Pe515cp at a m.o.i. of 5 or mock-infected with PBS and overlaid with maintenance medium. At 24 h, the medium overlay was removed and the cells detached using a cell scraper. Cells were washed twice with a 1:10 dilution of TNE buffer (100 mM Tris, 500 mM NaCl, and 10 mM EDTA, pH 8.0) and solubilized in TNE buffer containing 50  $\mu\text{M}$  phenylmethylsulfonyl fluoride (PMSF), 0.1% sodium dodecyl sulfate (SDS), and 0.5% NP40 (TNE lysis buffer). Cell lysates were adsorbed overnight at 4°C with 10% fixed staphylococcus (Protein A; Aldrich) in TNE lysis buffer, clarified by centrifugation, and stored at  $-80^\circ\text{C}$  until required. Serum (20  $\mu\text{l}$ ) was mixed with 100  $\mu\text{l}$  of cell lysate and 250  $\mu\text{l}$  of TNE lysis buffer and mixed overnight at 4°C with continuous rotation. Washed, immobilized Protein G ((80  $\mu\text{l}$ ; Aldrich) was added and the admix incubated for a further 4 h at 4°C. The immunosorbent was washed once with TNE lysis buffer and then four times with a 1:10 dilution of TNE buffer. Each sample was suspended in 30  $\mu\text{l}$  of electrophoresis sample buffer, resolved under re-

ducing conditions, and transferred to ECL-nitrocellulose membrane as described for the preparation of nitrocellulose-bound antigen. After blocking the membrane with 5% (w/v) semiskimmed milk in PBS containing 0.1% (v/v) NP40, the membranes were probed and processed for ECL visualization of proteins according to the manufacturer's instructions (Amersham Life Science, Buckinghamshire, UK) using a 1:10,000 dilution of 2331-B as the primary antibody and 1:10,000 streptavidin-HRP.

### Neutralization assays

Neutralizing antibody was determined in an immunoperoxidase microplate assay (Smith *et al.*, 1988). Test sera were heated for 90 min at 60°C to inactivate any persistent virus that was present. Sera were diluted in a twofold series and 50  $\mu\text{l}$  of diluted serum was mixed with an equal volume of medium containing 100 TCID<sub>50</sub> of virus. The antibody-virus admix was incubated for 60 min at 37°C and then 50  $\mu\text{l}$  transferred to the wells of a 96-well plate containing MDBK cells at 90–95% confluence. Cells and antibody-virus admix were incubated for 90 min at 37°C, after which the antibody-virus admix was discarded and the cell monolayer washed once with warm PBS. Wells were filled with 250  $\mu\text{l}$  maintenance medium and incubated for 5 days at 37°C, washed three times with warm PBS, and air-dried. Cell monolayers were fixed with PBS containing 20% acetone and 0.02% BSA for 30 min at room temperature, after which the fixative was discarded and the cell monolayers allowed to thoroughly air-dry. The presence of virus in fixed cells was visualized by immunoperoxidase staining using 2331-B as the BVDV probe.

### Lymphoproliferation assays

PBMC ( $10^5$ /well) were cultured in triplicate with antigen in 0.2 ml complete RPMI in 96-well round-bottom plates for 6 days at 37°C with 5%  $\text{CO}_2$ /95% air. Plates were pulsed with 7.4 kBq/well of (*methyl*- $^3\text{H}$ )-thymidine for the last 18 h of the assay and the cells were harvested onto glass-fiber filter mats using a Skatron harvester. Incorporation of radiolabel was determined using a Beckman 1205 scintillation counter. Results are expressed as the geometric mean of c.p.m. for replicate cultures.

In some experiments, live Pe515ncp virus was added to proliferation assays at 0, 0.05, 0.5, 5, or 20 m.o.i. to determine whether the presence of live virus affected the ability of PBMC to respond to cell-sonicate antigens.

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